

ISOLATION AND MOLECULAR CHARACTERIZATION OF *Brucella abortus* AND *Brucella melitensis* FROM SAMPLES RECEIVED BY THE REGIONAL VETERINARY LABORATORY, BUKIT TENGAH, MALAYSIA

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ABSTRACT. A study was carried out to report the phylogenetic analysis of *Brucella abortus* and *Brucella melitensis* by using molecular techniques from samples submitted to the Regional Veterinary Laboratory, Bukit Tengah. In this study, identification and genetic characterization of *Brucella* isolated samples using molecular analysis based on IS711 sequence between local isolates and foreign countries accesses in GenBank was done successfully. A total of 31 samples were isolated for *Brucella* species and then were amplified by PCR, directly sequenced and compared genetically to published sequences which were obtained from GenBank. The most common *Brucella* species that was found in both bovine (76.5%) and caprine (85.7%) through diagnostic samples in Regional Veterinary Laboratory, Bukit Tengah, was *Brucella melitensis*. PCR and sequencing were confirmed positive with 76.5% for *Brucella melitensis*, 23.5% for *Brucella abortus* and 23.5% for mixed infection from the total of 17 bovine samples. In caprine, the detection of *Brucella melitensis*

and *Brucella abortus* showed 85.7% and 21.4% respectively meanwhile total mixed infection showed 21.4%. These clustering between local isolates of *Brucella melitensis* were phylogenetically related to other Asian countries such as Singapore, Yemen and Saudi Arabia. The Neighbour Joining Analysis clustered the *Brucella abortus* local isolates for both bovine and caprine were most closely related to India, Iran, Italy and USA. Interestingly, all the isolates within Malaysia have a close relationship (>95%) with the low level of genetic diversity. When local isolates are compared to GenBank data, it gives an indication on the possible sources of these infections. Eventually, it will improve the import and export policies to control brucellosis in Malaysia.

Keywords: *Brucella abortus*, *Brucella melitensis*, caprine, bovine, phylogenetics tree

INTRODUCTION

Brucellosis is a zoonotic and infectious disease of livestock with worldwide

distribution (Corbel, 1997). The disease is manifested by abortion and infertility and caused by *Brucella* species which are gram-negative and facultative intracellular bacteria. There is generally a dearth of literature on brucellosis in Malaysia (Bahaman *et al.*, 2007). The organism was first isolated in bovine (*Brucella abortus*) in 1950. Later the infection was found to be more widespread than was envisaged at first (Joseph, 1987). To control the infection in our country, test and slaughter along with vaccination of bovine was carried out which somehow succeeded in controlling bovine brucellosis. Meanwhile *Brucella melitensis* case was first reported in caprine in 1994 in Malaysia (Moktar *et al.*, 1995).

In Malaysia, serological detection of *Brucella* species is done mainly by using Rose Bengal plate agglutination (RBPT) and complement fixation test (CFT) assay (Maged *et al.*, 2011). PCR based detection assay become prevalent in the last decade because they are specific, extremely sensitive and rapid (Brikenmeyer and Mushahwar, 1991). PCR assay using specific primers is the method of preference in order to obtain specific and sensitive output for both *Brucella melitensis* and *Brucella abortus* detection. In recent years, the application of molecular technologies has improved the information on the *Brucella* species and able to determine the relationship between available genotypes in the region.

Furthermore, studies on isolation and molecular characterization of *B. melitensis*

from seropositive caprine in Peninsular Malaysia by Bamaiyi in 2012 revealed that *B. melitensis* isolates were phylogenetically related to other isolates from India, Iran, and Israel but most closely related to isolates from Singapore. Other than that, Ratnasari R. (2013) reported that the homology and phylogenetic analyses of nucleotide sequence of *B. abortus* isolates from South Sulawesi are closely related to isolates from India and France, whereby *B. abortus* isolate from East Nusa Tenggara is closely related to isolate from South Sulawesi and India. To date there is scarce information on the current status of brucellosis with respect to molecular characterization. The purpose of this study was to identify and genetically characterize *Brucella* isolated samples using molecular analysis based on IS711 sequence as a targeted gene for *Brucella* species between local isolates and foreign countries accesses in GenBank.

MATERIALS AND METHOD

A total of 31 samples comprises reproductive tracts and organs of bovine and caprine origin from different region in Peninsular Malaysia were isolated for *Brucella* species by the Bacteriology Section, Regional Veterinary Laboratory, Bukit Tengah. Approximately 1 g sample was homogenized into 2 ml of phosphate buffered saline (PBS) with a ratio of 1:2 using mortar and pestle. The homogenized mixture was streaked onto serum dextrose agar with horse serum for four days at 37 °C with both aerobic and anaerobic

conditions. Suspected colonies based on morphology and microscopic examination was further subcultured for another two days to obtain pure isolates. Then, the isolates were confirmed biochemically as *Brucella* species according to the standard protocols which include triple sugar iron agar (TSI), sulfide-indole-motility (SIM), urease test, oxidase test, catalase test, nitrate reduction test, and glucose oxidative fermentative (OF) test. The isolated *Brucella* strains were extracted using a commercial kit (Qiagen DNeasy Blood and Tissue Kit) to elute DNA.

The DNA was optimized by using PCR parameters with published primers designed by Halling and Bricker (1994) for *Brucella abortus* and Redkar (2001) for *Brucella melitensis* with different forward and reverse primers, targeting the *Brucella*-specific IS711 sequence. The PCR parameters were optimized with annealing temperature of 58 °C, 1× PCR buffer, Mg²⁺ concentration of 2.5 mM, dNTPs concentration of 200 μM, taq polymerase concentration of 0.5 U per reaction (Promega Inc. USA). DNA amplification reaction was performed at these conditions; incubation at 95 °C for 5 mins denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, elongation at 72 °C for 30 s and extension at 72 °C for 5 mins with amplification of 35 cycles. The singleplex assay was conducted to amplify *Brucella abortus* and *Brucella melitensis* by using following primers:

5'-GACGAACGGAATTTTCCAATCCC-3'
(forward);

5'-TGCCGATCACTTAAGGGCCTTCAT-3'
(reverse) and

5'-CATGCGCTATGTCTGGTTAC-3'
(forward);

5'-AGTGTTCGGCTCAGAATAATC-3'
(reverse) respectively.

The *Brucella* species reference cultures were obtained from Universiti Putra Malaysia (UPM). The expected product size for *Brucella abortus* was 498 bp and *Brucella melitensis* was 252 bp. The amplified PCR products were visualized by gel electrophoresis with 2% agarose (OIE Terrestrial Manual, 2009). Confirmation of the PCR product was done by sending to First BASE Laboratories Sdn. Bhd. for PCR purification and further with nucleotide sequencing which was performed by Applied Biosystems 3730×1 DNA Analyzer. The sequence results were aligned and compared with other available sequence at NCBI BLAST (Basic Local Alignment Search Tool), and the phylogenetic tree was created with Neighbour Joining Method by using the Software MEGA 5.

RESULTS

About 31 samples of bovine and caprine in Regional Veterinary Laboratory Bukit Tengah were diagnosed for brucellosis in particular for *Brucella abortus* and *Brucella melitensis* based on PCR technique as per

Table 1. The overall data of molecular PCR detection for *Brucella abortus* and *Brucella melitensis* from samples received by Regional Veterinary Laboratory Bukit Tengah.

Species	Total of samples	Detection of <i>Brucella abortus</i>		Detection of <i>Brucella melitensis</i>		Total of Mixed infection
		Positive (+)	Negative (-)	Positive (+)	Negative (-)	
Bovine	17	4 (23.5%)	13 (76.5%)	13 (76.5%)	4 (23.5%)	4 (23.5%)
Caprine	14	3 (21.4%)	11 (78.6%)	12 (85.7%)	2 (14.3%)	3 (21.4%)

Table 1. Among the molecular detection, *Brucella melitensis* (76.5%) was detected higher than *Brucella abortus* (23.5%) in bovine. Meanwhile, from the total sample of 17 bovine samples, approximately 23.5% showed mixed infection. In caprine, the molecular detection of *Brucella melitensis* (85.7%) showed higher than *Brucella abortus* (21.4%). Other than that, total mixed infection in caprine showed 21.4%. The total of mixed infection results was obtained by totaling up the both *Brucella abortus* and *Brucella melitensis* detection from the same infected samples.

The amplification of targeted DNA by using specific primers for both *Brucella abortus* and *Brucella melitensis* was electrophoresed and visualized by UV light as per on Figures 1 and 2. An annealing temperature of 58 °C was used to increase the specificity to obtain targeted DNA. In this observation, PCR technique was able to detect *Brucella abortus* at 498 bp (Bricker and Halling, 1994) and *Brucella melitensis* at 252 bp (Redkar, 2001).

About 25 of *Brucella melitensis* DNA samples and 7 of *Brucella abortus* DNA samples were sent for nucleotide sequencing and was performed with 3730x1 DNA Analyzer software. This program was used to match in order to obtain full length of nucleotide sequence. The sequence of 498 bp PCR product of *Brucella abortus* local isolates and 252 bp PCR product of *Brucella melitensis* local isolates are showed in Figures 3 and 4.

The phylogenetic tree revealed that our *Brucella melitensis* isolates are genetically related to lineages topotypes from other parts of Asia namely Singapore (GQ479519), Yemen (KF730265), and Saubi Arabia (GQ479519). Figure 5 explained the similarity of the *Brucella melitensis* isolates from samples received by Regional Veterinary Laboratory, Bukit Tengah, with others from other countries using the Neighbour Joining Method. Meanwhile, the Neighbour Joining Analysis clustered the *Brucella abortus* local isolates within a large cluster with

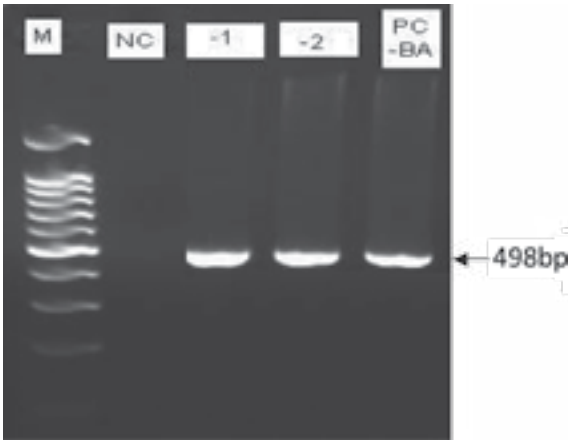


Figure 1. The amplification of targeted DNA by using specific primers with expected band (498bp) for *Brucella abortus* local isolates (1) and *Brucella abortus* local isolates (2) and *Brucella abortus* reference strain (PC-BA), (NC) is Nuclease Free Water (Negative Control) and (M) is 100 bp DNA marker.

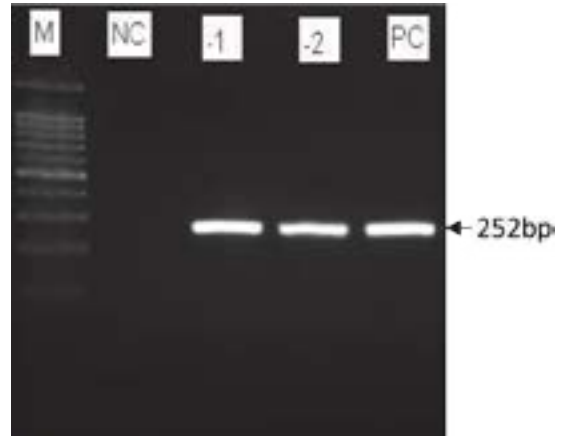


Figure 2. The amplification of targeted DNA by using specific primers with expected band (252 bp) for *Brucella melitensis* local isolate (1) and *Brucella melitensis* local isolate (2) and *Brucella melitensis* reference strain (PC), (NC) is Nuclease Free Water (Negative Control) and (M) is 100 bp DNA marker.

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TTTTGACGAACGGGATTTTTCCAATCCCATCGTTT
CCGTTTCACTTGGCCTGCCGGCAACGTTTCAGTTT
GGCGGAATGAAACGGACGGACCCGATCACCAAATA
TATCCTGCACCATGGCGACGTGGTTGTCTGGGGCG
GGCCGTGCGGGCTTTTCTATCACGGTATTCTACCA
TTGAAGTCTGGCGAGCATGAGCGGCTGGGGCCGTT
TCGGCTTAATCTGACCTTCCGAAAGGCGTTCTAGG
GCGTGTCTGCATTTAACGTAACCAGATCATAGCGC
ATGCGAGATGGACGAAACCCATGAATGCGGTCAAT
GTTTTCTCGCATCGCAGCGCAATACGACGATAGCG
TTTCAACTTGTTAAAAAAGCATTCAATCTGATGGC
GTTTCTTGTACAGCCTCCAGTCGATTGTTGGGGCA
CTGGAACGTGTTGGATTGACCTTGATCTGAGCCGT
TGCCTTGAGATCGCTGGCAATGAAGGCCCTTAATG
GATCGGCAA
    
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Figure 3. Nucleotide sequence of *Brucella abortus* isolate from samples received by Regional Veterinary Laboratory, Bukit Tengah.

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TCATGCGCTATGTCTGGTT
ACGTTGAATGCAGACACGC
CCTAGGGGTGAATCTGGAA
ATTGTCAGAAAGACAGTGC
TTCGTACGCTAGAGCGCT
CGCTGCCATACTTGCAACA
GTGACAGCGATAAATTGCCG
TTATTGGCTGGTGGCAGGG
CGAAGATTGGCGGGTAAGC
TATTCCAATCTCGCTATTG
TTAATGGCGTCTATTGGAT
ATTACTGCTCTACCTTCTG
TGGATTATTCTGAGCCGAA
ACACT
    
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Figure 4. Nucleotide sequence of *Brucella melitensis* isolate from samples received by Regional Veterinary Laboratory, Bukit Tengah.

profiles from caprine and bovine were mostly genetically related to isolates from India (FM162593), Iran (DQ845342), Italy (AJ314585) and USA (AF148682) as per Figure 6. The phylogenetic trees were constructed by using Neighbour-Joining Method. The phylogenetic tree is a two dimensional of a species graphic that shows relationship among the gene sequences. If the nucleotide sequence from

two different organism are similar, they were indirect to be derived from common ancestor (Dharmayanti, 2011)

DISCUSSION

The PCR amplification was successfully done and able to detect specific gene product of *Brucella abortus* at 498 bp according to Bricker and Halling in 1994 and *Brucella*



Figure 5. Dendrogram of *Brucella melitensis* isolates from samples received by Regional Veterinary Laboratory, Bukit Tengah, and other isolates from other countries using the Neighbour Joining Method.



Figure 6. Dendrogram of *Brucella abortus* isolates from samples received by Regional Veterinary Laboratory, Bukit Tengah, and other isolates from other countries using the Neighbour Joining Method.

melitensis at 252 bp according to Redkar in 2001. The testing indicated several results including detection of *Brucella abortus*, *Brucella melitensis* and mixed infection of brucellosis. Yet, brucellosis in caprine is primarily caused by *Brucella melitensis* and rarely by *Brucella abortus* (Luchsinger and Anderson, 1979). In regions where *Brucella melitensis* is prevalent in caprine, bovine may become infected with the disease from them. Moreover, in some countries, like in Southern Europe and Western Asia, where bovine are kept in close association with caprine also can cause infection by *Brucella melitensis* (OIE Terrestrial Manual, 2009). It has not been established whether *Brucella melitensis* can maintain itself indefinitely

in a bovine population in the absence of infected caprine (WHO 2001). Commonly, *Brucella abortus* is not among principal pathogenic *Brucella* species that isolated from caprine, since the caprine appears to be most susceptible to *Brucella melitensis*, but may also be infected with *Brucella abortus*, which causes occasional infections in caprine resulting in abortions (Carter 1995).

Analysis on phylogenetic study of the current available isolates was done for molecular characterization of *Brucella abortus* and *Brucella melitensis* in each case to determine the probable origin of the organism and identify the similarity of the organism worldwide with the current isolates in Malaysia. The analyzed gene

sequences revealed that *Brucella melitensis* local isolates were genetically related to lineages topotypes to Singapore, Yemen, and Saudi Arabia. Behl (2003) reported that Saudi Arabia caprine showed less genetic diversity when compared with some of the Indian caprine. This indicates a common ancestral evolutionary origin of the isolates from caprine (Hall, 2001).

It is also known that geographical locations play an important role in relationship between isolates with isolates from similar regions tending to be more closely related (Hall, 2001). Besides that, the livestock breeds from neighbourhood countries such as Singapore prone to get infected from similar sources due to import-export activities (Bamaiyi, 2012). The transcontinental spread of *Brucella melitensis* could therefore have marked the beginning of the diversification of the *Brucella melitensis* strains. The global spread of *Brucella melitensis* may have occurred following these ancient trading routes, perhaps through infected caprine or their milk derivatives (Kim-Kee Tan *et al.*, 2015). Caprine remain the main source of infection, but *Brucella melitensis* in bovine has emerged as an important problem in some southern European countries, Israel, Kuwait, and Saudi Arabia. *Brucella melitensis* infection is particularly problematic because *Brucella abortus* vaccines do not protect effectively against *Brucella melitensis* infection meanwhile the *Brucella melitensis* vaccine Rev. 1 has not been fully evaluated for use in bovine (Michael J. Corbel, 1997). In Al-Garadi *et*

al. (2011) reported that *Brucella melitensis* isolates in caprine are closely related to each other in Malaysia. The paper from Bamaiyi in year 2012 also added the evidence of the similarity relationship among the *Brucella melitensis* of caprine in Peninsular Malaysia based on molecular characterization of the local isolates.

Meanwhile, the Neighbour Joining Analysis clustered the *Brucella abortus* local isolates within a large cluster with profiles from caprine and bovine were mostly genetically related to isolates from India, Iran, Italy and USA. The *Brucella* isolates was composed of strains from India, Iran, Italy and USA. This finding related to the facts that the bovine samples investigated in this study were mostly animals imported from India and Pakistan. Therefore, it is highly suggested that the *Brucella abortus* isolates from this study originates from the animals country of origin. In the published literature, isolates within Malaysia have a close relationship (>95%), with level of variations common in geographically proximate samples (Nagalingam *et al.*, 2012) as point of transformations may occur due to factors such as transhumance, imports-exports, breed, mutation and species variability.

CONCLUSION

From the sequencing analysis results, were able to sequence accordingly both *Brucella abortus* and *Brucella melitensis* isolates. The genetic homology and diversity between the local isolates received by RVLBT with

reference strains from the GenBank helps to identify the possible origin source of infection into the country. Further analysis on phylogenetic study of overall isolates in Malaysia is necessary for molecular characterization of *Brucella abortus* and *Brucella melitensis* in determining the probable origin of the organism and to identify the similarity or diversity with the other *Brucella* species worldwide. It is also important in establishing the isolates role in the epidemiology of brucellosis and helps to control and eradicate brucellosis in Malaysia.

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